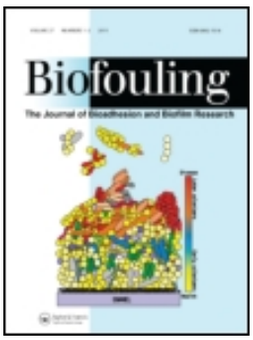

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ADHESION OF BIOFILMS TO INERT SURFACES: A MOLECULAR LEVEL APPROACH DIRECTED AT THE MARINE ENVIRONMENT

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Protein/ligand interactions involved in mediating adhesion between microorganisms and biological surfaces have been well-characterized in some cases (*e.g.* pathogen/host interactions). The strategies microorganisms employ for attachment to inert surfaces have not been so clearly elucidated. An experimental approach is presented which addresses the issues from the point of view of molecular interactions occurring at the interface.

KEYWORDS: fouling, molecular interactions, adhesin, marine

FEASIBILITY AND POTENTIAL FRUITS OF THE APPROACH

Communities of bacteria which have colonized a surface present a fascinating subject for microbiological investigation. The conceivable extent of the organizational complexity of these surface-associated communities has only recently become apparent. For example, cells in a biofilm are typically embedded in a matrix of extracellular polymeric substances (EPS) (Costerton *et al.*, 1987; Cooksey, 1992) commonly referred to as "slime". Recent studies suggest that this heterogeneous network of bioploymers may serve in some cases to optimize biofilm architecture such that oxygen transport (DeBeer *et al.*, 1993) or nutrient utilization (Wolfaardt *et al.*, 1994) are facilitated. There is even some hint that for some biofilms the three dimensional lattice can metamorphose in response to toxins (Korber *et al.*, 1994). Studies reveal that bacteria adapt by utilizing networks of regulatory genes which respond to a host of environmental stimuli (Parkinson, 1993; Deretic *et al.*, 1994; Loewen & Hengge-Aronis, 1994). If these findings are viewed in the light of evidence for cell-to-cell communication, effected at the level of genetic regulation (Passador *et al.*, 1993; Piper *et al.*, 1993), for an expanding number of bacterial species (Klotz, 1993), the implication that there may be some points for comparison of many biofilms with specialized tissues in multicellular organisms becomes reasonable (Jackson *et al.*, 1986; Kaiser & Losick, 1993).

Despite the complex nature of biofilms, a molecular level (biochemical) approach toward understanding their adhesion to inert surfaces should be tractable. The framework

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for analyzing and understanding adsorption of biomolecules to engineered materials has been established, primarily in literature concerning blood compatibility (Andrade, 1985; Bohnert & Horbett, 1986; Brash & Horbett, 1987; Krisdhasima *et al.*, 1993; Haynes & Norde, 1995). Methods for EPS isolation and purification are available (Troy, 1979; Read & Costerton, 1987; Henningson & Gudmestad, 1993). Adhesion inhibition assays conventionally used to investigate specific adhesion mediated by protein/ligand interactions (Jann & Jann, 1993) may be applicable in some cases (Merker & Smit, 1988; Quintero & Weiner, 1995). Biomolecules involved in attachment of cells to inert surfaces have been classified according to general type (proteins or polysaccharide) by utilizing proteolytic enzymes and surfactants (Paul & Jeffrey, 1985). Detailed biochemical characterization of adhesin remnants left by displaced microorganisms (''footprints'') (Neu, 1992) is becoming increasingly feasible as sophisticated surface analysis techniques are adapted for analysis of biological samples (Mantus *et al.*, 1993). In at least one case, genetic determinants of a non-specific holdfast have been characterized (Yun *et al.*, 1994). The fruits of a molecular level approach can be relevant to understanding particular aspects of even extremely complex surface-associated ecosystems such as climax marine fouling communities; *i.e.* it can be expected that these communities will be anchored to the substratum *via* contributions from at least the most adhesive biomolecules expressed by the primary colonizers. This is a testable hypothesis since exchange reactions at interfaces can be characterized (Koltisko & Walton, 1985).

At a molecular (reductionist or biochemical) level the adhesive relationship between a developing fouling community and the inert surface can be simplified to an investigation of interfacial interactions among adsorbed and impinging biopolymers. A number of interactions between solution phase and preadsorbed biomolecules are possible. These include displacement (or exchange) (Vroman & Adams, 1986), intercalation to unoccupied sites, which may promote ''pinning'' of the preadsorbed species (Johnson & Granick, 1992), or binding to moieties on the preadsorbed film (Suci & Geesey, 1995). There may be no direct, simple correspondence between adhesive properties and interactions of isolated biomolecules, and the molecular interactions which mediate adhesion of a fouling community. For example, the simplest fouling community consists of single cells attached to a substratum. Even for this simple case, the molecular interactions of an adhesin with a surface can be altered by the tether or anchor to the cell in a number of ways. For example functional groups involved in securing the adhesin to the cell will be less likely to participate in binding to the surface; the stereochemistry of the adhesin may be altered, reducing the entropy gain upon binding. However, despite the possible caveats, adsorption behavior of biomolecules has been positively correlated to whole cell adhesion behavior and used to identify potential adhesins (Pringle & Fletcher, 1986; Bidle *et al.*, 1993).

Less reductionistic frameworks for interpreting adhesion behavior are available (Busscher *et al.*, 1989). The application of DLVO theory, which can adequately account for observed cell adhesion behavior in some cases, essentially ignores individual molecular interactions (Van Loosdrecht *et al.*, 1989). Similarly, some cell adhesion behavior can be predicted on the basis of cell-surface hydrophobicity (Rosenberg & Kjelleberg, 1986; Stenström, 1989). A molecular level approach may provide a basis for interpreting patterns of adhesion behavior which do not conform easily to the predictions of either of these more global schemes (McEldowney & Fletcher, 1986). In addition, when combined with isolation and biochemical analysis of EPS components, it allows examination of a number of related questions. Have certain biomolecules evolved to serve as non-specific adhesins (Yun *et al.*, 1994)? Do particular classes of biomolecules mediate adhesion to inert surfaces having similar properties (Paul & Jeffrey, 1985)? Is

molecular architecture crafted by organisms to promote either adhesion to, or detachment from (Wrangstadh *et al.*, 1986) inert surfaces?

MARINE CONDITIONING FILMS

The interfacial interactions between biomolecules and the surface which were outlined above will probably begin before the first encounter between microbes and an engineered surface placed in the marine environment. It is well known that biomaterials in contact with blood will be coated rapidly with intrinsic proteins (Gendreau *et al.*, 1981). Similarly, adsorption of salivary components onto oral surfaces creates the organic biopolymer film (the pellicle) to which bacteria eventually attach (Kolenbrander & London, 1993). The macromolecular content of the sea is considerably more dilute than that of blood or the oral cavity. Estimates of dissolved organic carbon (DOC) are in the range of 0.5 to 1.0 mg l^{-1} in the open sea and can be ten times higher in coastal waters (Thurman, 1985; Benner *et al.*, 1992). However, organic "conditioning" films apparently form quickly on materials placed in seawater (Loeb & Neihof, 1975; 1977; Kristoffersen *et al.*, 1982).

Definitive biochemical characterization of marine conditioning films is lacking, except for a study suggesting incorporation of proteinaceous material (Baier *et al.*, 1983), and a study indicating that the composition of the film is site specific (Little, 1985). However, there is indirect evidence supporting the supposition that the films may consist of a large proportion of biopolymeric material regardless of the site. Much of the organic material in the sea is in the form of colloids which range in size from 5–200 nm (Wells & Goldberg, 1994). There is evidence that a substantial portion of the carbohydrates are in the form of polysaccharides (Pakulski & Benner, 1994). The total amino acid content ranges from 0.06 to 0.24 mg l^{-1} with the combined fraction being on average five times as high as the free amino acids on a mass per volume basis (Thurman, 1985). This suggests that many of the amino acids are also incorporated into biopolymers. Polymers tend to have high affinity isotherms (Cohen Stuart *et al.*, 1982). This implies that, given enough time, even very dilute solutions will saturate the surface binding sites. This prediction has been verified experimentally for adsorption of bovine serum albumin (BSA) on stainless steel (Van Enckevort *et al.*, 1984).

EXPERIMENTAL APPROACH

A number of biomolecules have been selected as model compounds, *viz.* mussel adhesive protein (MAP) from *Mytilis edulis*, an adhesive polysaccharide (fraction 2 polysaccharide (fr2ps)) from the marine bacterium, *Hyphomonas* MHS-3 (MHS-3), alginate from *Macrocystis pyrifera* and the globular blood protein, bovine serum albumin (BSA). Interactions between these biomolecules and germanium (Ge), polystyrene (PS), and poly (octadecyl methacrylate) (POMA) surfaces in contact with synthetic seawater are being investigated.

Both MAP and BSA adsorb tenaciously to both hydrophobic and hydrophilic substrata. MAP constitutes the resin of a natural thermoset adhesive with which *M. edulis* anchors itself to a variety of substrata (Waite, 1990), and is sold commercially as a coating to enhance cell adhesion (Benedict & Picciano, 1987; Notter, 1988). BSA is often used to block nonspecific binding sites (Goding, 1983), and in many cases reduces cell attachment (*e.g.* Pratt-Terpstra *et al.*, 1987; Tamada & Ikada, 1993). Evidence has been

presented that fr2ps is a polysaccharide adhesin which mediates attachment of MHS-3 to (at least) hydrophilic substrata (Quintero & Weiner, 1995; Frølund *et al.*, 1996). Alginate is a linear copolymer composed of the uronic acids α-L-guluronic and β-D-mannuronic acid (Cotrell & Kovacs, 1977). Derivatives of this polysaccharide are the primary exopolysaccharides produced by mucoid strains of *Pseudomonas aeruginosa* (Evans & Linker, 1973). Uronic acids constitute a significant fraction of the EPS of several marine pseudomonads (Uhlinger & White, 1983; Christensen *et al.*, 1985) and are a common component of extracellular and cell surface biopolymers of most marine bacteria studied to date (Sutherland, 1980; Christensen, 1989).

Thus far, investigations have been confined to a relatively simple system, *i.e.* interaction of a solution phase polysaccharide with a preadsorbed (irreversibly bound) protein film coating a submerged substratum. The complementary experiment has also been performed. *i.e.* exposure of a preadsorbed polysaccharide film to solution phase protein. These types of dual adsorption experiments are particularly compatible with the technique of attenuated total reflection Fourier transform infrared (ATR/FT-IR) spectrometry (Ishida & Griffiths, 1993). The term "irreversibly bound" requires explanation. Although it has a precise thermodynamic meaning (Norde *et al.*, 1986), it has been used (in an operational sense) to describe an adsorbed species (usually macromolecular) which cannot be rinsed from a surface (Bohnert & Horbett, 1986). The term is used here in the latter (less precise) sense.

Alginate does not adsorb with great affinity to Ge (Ishida & Griffiths, 1993). However, preconditioning Ge substrata with MAP enhances alginate adsorption (Suci & Geesey, 1995). The driving force for adsorption in synthetic seawater (pH 8) appears to be primarily electrostatic, conferred by interaction of protonated lysine residues of the MAP and the negatively charged carboxylate functionalities of the alginate. As expected, conditioning Ge with BSA, which has an isoelectric point (IEP) of approximately 5, does not enhance alginate adsorption. The data suggest that once the alginate has penetrated the electrostatic double layer, it forms additional bonds with MAP which involve pyranose ring atoms. According to the FT-IR diagnostic, the interaction of alginate with preadsorbed MAP was not affected by the underlying substratum (Ge *vs* PS); both the type(s) of bonding, and the extent of adsorption per surface coverage of MAP were the same regardless of whether the underlying substratum was Ge or PS.

Whereas MAP enhances alginate adsorption to Ge, conditioning Ge with MAP, BSA or intrinsic MHS-3 EPS proteins depresses adsorption of fr2ps, the putative MHS-3 adhesin (Suci *et al.*, 1995; Frølund *et al.*, 1996). The molecular characteristics of MAP and BSA are in most ways contrasting. MAP is rich in lysine and DOPA residues, has an open conformation (Williams *et al.*, 1989) and has an IEP above 10. BSA has ample secondary and tertiary structure, and has a net negative charge at pH 8 (Peters, 1985). Adsorption of fr2ps was reduced to a similar extent (compared to unconditioned Ge) on Ge conditioned with MAP and BSA, which suggests that electrostatic repulsion is not a dominant factor in excluding fr2ps from the conditioned substrata. Adsorption of MHS-3 cells to Ge conditioned with MAP or BSA was also depressed (Frølund *et al.*, 1996), implying that interactions characterized at the molecular level are relevant to the functioning of the adhesin *in situ*.

MHS-3 ADHESION TO PS AND POMA SUBSTRATA CONDITIONED WITH MAP

The question of whether adhesion-mediating substratum properties are "transferred" through a protein conditioning film has been posed by a number of investigators (*e.g.*

Pratt-Terpstra *et al.*, 1987; Schakenraad *et al.*, 1989; Busscher *et al.*, 1990; Ranieri *et al.*, 1993; Tamada & Ikada, 1993; Al-Makhlafi *et al.*, 1994). Substrata may exert their influence on cell adhesion in the presence of a preadsorbed film in the trivial sense, due to incomplete coverage of the original surface by the film, or the original substrata may select for different subsets of proteins from a mixture used to form the film (Tamada & Ikada, 1993). Alternatively, the substrata may influence properties of the adsorbed state of a protein (or proteins) in the film differently, thus inducing different film structures (Pratt-Terpstra *et al.*, 1987; Busscher *et al.*, 1990; Al-Makhlafi *et al.*, 1994). It is generally accepted that proteins which are irreversibly adsorbed alter their conformation to reach the final adsorbed state (Norde *et al.*, 1986; Haynes & Norde, 1995). Measurements indicate that different substrata induce different kinds of conformational changes in proteins (Bohnert & Horbett, 1986; Pitt & Cooper, 1988; Banovac *et al.*, 1994; Haynes & Norde, 1995). It has been proposed that the adsorbed protein film structure may be partially determined by the balance between cohesive (protein/protein) and adhesive (protein/surface) forces (Taylor *et al.*, 1994).

A detailed study of the absorbed film structure of MAP on PS and POMA using atomic force microscopy (AFM), X-ray photoelectron spectrometry (XPS) and ATR/FT-IR has been performed (Baty *et al.*, 1995). The adsorbed film exhibits markedly different surface topography on PS and POMA (Fig. 1). The fibrous network observed on the POMA surface indicates that the proteins have formed aggregates upon adsorption, while on the PS surface the largest resolvable structures approach the size of an individual mefp-1 molecule (the primary component of MAP). These results suggest that on the PS surface, protein/surface interactions dominate, while on the POMA surface, protein/protein interactions determine the supramolecular structure. XPS and ATR/FT-IR data are consistent with this interpretation. Recent studies indicate that the structural differences are preserved in the hydrated state (Baty *et al.*, unpublished).

It might be anticipated that these distinctly different adsorbed film structures would induce comparatively large differences in cell adhesion behavior. In the case of MHS-3 this prediction is not supported by the data. Preliminary results from ATR/FT-IR adsorption studies indicate that fr2ps adsorbs quite differently to unconditioned PS and POMA, but that these differences are mitigated by conditioning with MAP; the spectra are somewhat difficult to interpret due to the large background signal from the polymer films (data not shown). The results of MHS-3 whole cell adhesion assays are much less ambiguous (Fig. 2). It is apparent that the MAP conditioning film masks the underlying substratum properties, at least with respect to numbers of cells attaching within the 30 min exposure period.

It is well known that conditioning with BSA can inhibit adsorption of other biomolecules and reduce cell adhesion. (Note, however, that adsorption onto certain substrata can reverse this latter trend (Ranieri *et al.*, 1993)). At anionic and hydrophobic surfaces BSA is thought to assume a surface orientation which presents a negatively charged domain (one of three) to the aqueous phase (Andrade *et al.*, 1990). This surface orientation could be expected to exclude many bacterial strains which are generally considered to have net negatively charged cell envelopes. The reduced adsorption of fr2ps and reduced adhesion of MHS-3 cells to MAP requires a different explanation. MAP is rich with lysine residues which are protonated at the pH of the synthetic seawater (8.0). These lysine residues are apparently available for binding to solution phase biopolymers since they enhance alginate binding in seawater as described above.

The reduced cell adhesion on conditioned surfaces cannot be accommodated, at least in a simple way, within a framework of hydrophobic/hydrophilic tendencies. Although the extent of cell adhesion to POMA, PS and Ge surfaces appears to indicate a preference

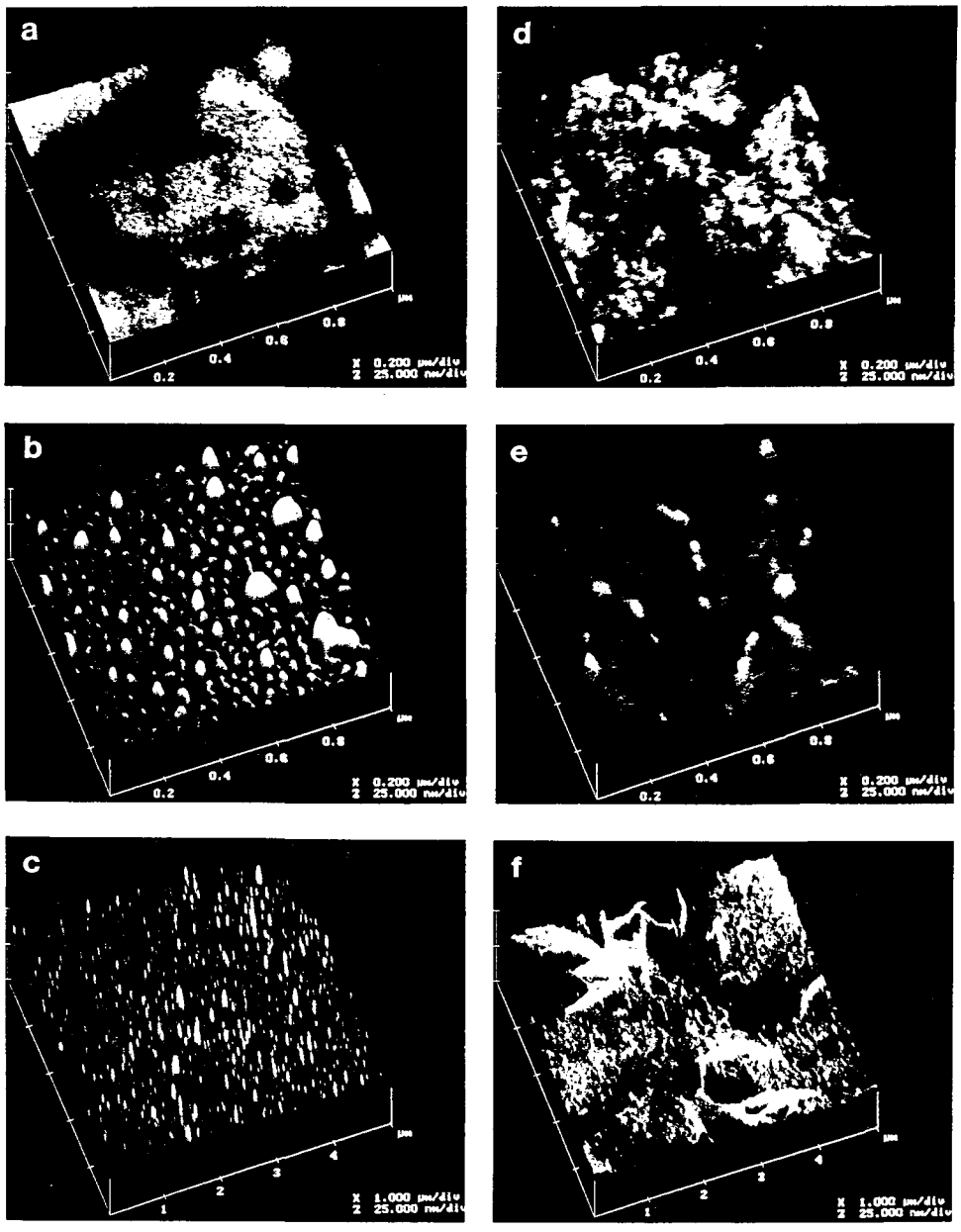

Fig. 1 a,d; b,e; c,f = AFM contour images, Underlying substratum is PS (left side) or POMA (right side). a = 1 μm × 1 μm area of PS before protein adsorption; b = 1 μm × 1 μm area of MAP adsorbed to PS; c = 5 μm × 5 μm area of MAP adsorbed to PS; d = 1 μm × 1 μm area of POMA before protein adsorption; e = 1 μm × 1 μm area of MAP adsorbed to POMA; f = 5 μm × 5 μm area of MAP adsorbed to POMA. Protein adsorption was performed in a 50 μg ml^{-1} solution of MAP for 60 min followed by a rinse using fluid displacement (Baty *et al.*, 1995; with permission, *J Colloid Interface Sci*). (see Color Section.)

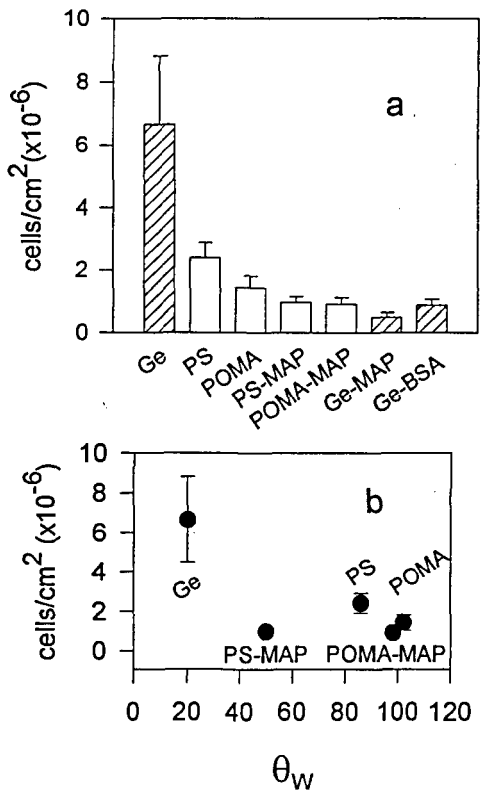

Fig. 2 a = adhesion of MHS-3 cells onto various substrata. Substrata conditioned with MAP or BSA are indicated by hyphenation (*e.g.* PS-BSA). Error bars are standard deviations. Cross hatched bars indicate data presented previously (Frølund *et al.*, *1996*). For new data (bars not cross-hatched) PS > POMA > PS > MAP = POMA-MAP, based on 2 sample t-test (0.95). Experimental details are described in Baty *et al.* (1995) and Frølund *et al.* (1996). Polymer films (PS and POMA) were spun cast onto Ge coupons. Preadsorption of MAP and BSA was for 60 min under static conditions. Substrata were exposed to washed MHS-3 cells for 30 min and then rinsed in synthetic seawater. Attached cells were stained and observed using epifluorescence microscopy. b = advancing water contact angles for some of the surfaces obtained using the sessile drop method *vs* number of adhered cells.

for hydrophilic substrata with unconditioned surfaces (Fig. 2b), the conditioned surfaces are exceptions to this trend. The high contact angle obtained for the POMA-MAP surface is thought to result from interaction of the water drop with portions of the substratum which are exposed when MAP coalesces into aggregates upon dehydration (Baty *et al.*, 1995; unpublished). If this interpretation is correct, then both the PS and POMA surfaces conditioned with MAP may exhibit similar hydrophilicities (mildly hydrophilic) in the hydrated state.

It may be that the exclusion of fr2ps from both BSA and MAP results from steric repulsion, a phrase which is used to explain protein exclusion from self assembled monolayers of oligo (ethylene oxide). These films can exclude both solution phase proteins (Prime & Whitesides, 1993) and whole cells (Lopez *et al.*, 1993). The effect has been compared to steric stabilization of colloids by adsorption of a hydrophilic polymer at the water/colloid interface (Prime & Whitesides, 1991). Steric repulsion between

adsorbed protein films can be measured using the surface-force apparatus (Nylander *et al.*, 1994), and the phrase has been invoked to explain the exclusion of methylated BSA from preadsorbed films of BSA (Staunton & Quiquampoix, 1994). Many proteins saturate the surface at monolayer coverage which means they exclude at least themselves from the interface (Malmsten, 1994; Nylander *et al.*, 1994). This exclusion occurs even though the adsorbed proteins might be expected to have altered conformations which would promote binding of their solution phase counterparts.

The similar extent of (reduced) cell adhesion on the PS and POMA surfaces conditioned with MAP suggest that the molecular interactions involved are unaffected by a pronounced difference in adsorbed (conditioning) film supramolecular structure. This result is reminiscent of the phenomenon of protein and cell exclusion reported for oligo (ethylene oxide) films, *i.e.* the effect is robust in the sense that the ethylene glycol chains need not be individually grafted to the surface to produce the exclusion, but can be graft-polymerized *via* plasma polymerization (Lopez *et al.*, 1992). This deposition process produces films with more chemical heterogeneity than self-assembled films (Johnston & Ratner, 1995). (Plasma polymerized films are normally highly cross-linked).

The analogy drawn above may be superficial when examined closely. The putative MHS-3 adhesin, fr2ps, is thought to be located in a diffuse capsular envelope on the mother cell (Quintero & Weiner, 1995). Mineral surfaces can participate in hydrogen bonding with both organic acids and bases (Thurman, 1985) and it has been postulated that MHS-3 capsular polysaccharide binds to substrata primarily through hydrogen bonding (Quintero & Weiner, 1995). In theoretical treatments of steric repulsion associated with oligo (ethylene oxide) films hydrogen bonding is not explicitly considered (Jeon & Andrade, 1991). This may leave an opening for some interesting studies.

SUMMARY AND CONCLUSIONS

In general biofilms are complex communities of interacting microbes. Despite this complexity a molecular level approach to investigating interactions which mediate adhesion of fouling communities to inert surfaces is tractable. Experimental methodologies and associated interpretative tools are available. The interface between the biofilm and the inert surface provides a clear focus for investigation of adsorption behavior of biomolecules. Such studies can yield useful information about adhesins involved, molecular architectures which may promote adhesive (or non-adhesive) interactions, and information about interactions between biomolecules at the interface.

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